Identification of Ferroptosis-related genes in sepsis-induced acute respiratory distress syndrome

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Article

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Abstract

Objective: The objective of this investigation was to examine the association between genes related to ferroptosis (FAGs) and acute respiratory distress syndrome (ARDS) induced by sepsis in individuals.

Methods: Microarray profiles (GSE332707) from the GEO database were utilized to screen for differential genes. FAGs were derived from three gene pools (KEGG database, NCBI, FerrDb database). The co-expression genes were obtained through the intersection of differential genes (DEGs) in GSE332707 and FAGs. Subsequently, hub genes were discerned by means of GO, KEGG, and PPI network analysis. The validation of these hub genes was carried out experimentally using the RT-qPCR technique and a separate dataset (GSE66890). Ultimately, target gene prediction was conducted through the utilization of GeneCard and StarBase 3.0.

Results: A total of 565 DEGs were identified between sepsis-induced ARDS and control samples, with 30 co-differential genes being detected. Then, the protein interaction network of 30 co-differential genes unearthed 5 hub genes (CTSB, LCN2, ZFP36, KLF2, and IRF1). Validation of the 5 hub genes was performed using RT-qPCR and GSE66890, which confirmed LCN2 as a potential prognostic candidate gene. The hsa-miR-374b-3p emerged as the most strongly supported candidate miRNA of LCN2.

Conclusion: Based on our findings, we conclude that LCN2, a potential biomarker associated with FAGs, may play a role in the pathogenesis of sepsis-induced ARDS.

Introduction

The lungs are highly susceptible to the effects of airborne toxicants, which can induce inflammation in this vital organ due to its constant gaseous exchange function. A multitude of factors, including sepsis, mechanical ventilation, and ischemia-reperfusion injury, can trigger the onset of acute respiratory distress syndrome (ARDS) (1). Sepsis-induced ARDS is a commonly occurring severe complication that significantly contributes to high mortality rates, reaching up to 50% (2). This condition is characterized by a pathological process that involves the development of pulmonary edema, thickened alveolar septa, and a devastating hyperinflammatory response. Despite extensive research, the mechanisms underlying sepsis-induced ARDS remain incompletely understood.

The crucial role of the innate immune system in the pathogenesis of sepsis-induced ARDS has been well established (3). Inflammation, oxidative stress, and apoptotic are the most influencing pathogenic mechanism in the development of ARDS (4). Recent research has highlighted the pivotal role of ferroptosis in the development of ARDS and uncontrollable lung inflammation (5, 6). The activity of Glutathione peroxidase 4 (GPX4) has been observed in the parenchyma cells of mice infected with mycobacterium tuberculosis, indicating a potential protective role for GPX4 in the lungs. Additionally, there was a significant increase in lipid peroxide production in these cells (7). In mouse models of lipopolysaccharide (LPS)-induced ARDS, the deletion of free iron from bronchial epithelial cells and the downregulation of Solute Carrier Family 7 Member 11 (SLC7A11) and GPX4 expression resulted in
increased ferroptosis. But pretreatment with a ferroptosis inhibitor was found to provide protection against lung injury in mice, indicating that ferroptosis may play a significant role in the onset and progression of ARDS caused by LPS (8). Furthermore, it has been suggested that ferroptosis may be implicated in sepsis-induced ARDS in a rabbit model, potentially through the modulation of the nuclear factor erythroid2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway (9). Besides, the inhibition of ferroptosis in LPS-induced ARDS by panaxydol is potentially linked to the upregulation of the Kelch-like ECH-associated protein 1 (Keap1)-Nrf2/heme oxygenase-1 (HO-1) pathway, as evidenced by prior research (10). Collectively, these findings suggest that ferroptosis may serve as a pivotal factor in the development of sepsis-associated ARDS.

Ferroptosis is a recently discovered mode of programmed cell death that arises from iron overload and lipid peroxidation, distinguishing it from other conventional forms of programmed cell death. Previous investigations have primarily concentrated on the involvement of ferroptosis in chronic ailments, such as cancer (11). However, recent literature suggests that it also plays a pivotal pathogenic function in the acute physiological responses linked to lung injuries (5, 10). To conduct a comprehensive literature review, we conducted a search on PubMed utilizing the keywords "ferroptosis", "sepsis", and "lung injury". A total of 19 relevant studies were identified, with a majority of them concentrating on signaling pathways that are involved in the regulation of ferroptosis. However, there is a dearth of research studies that focus on the crucial ferroptosis-related genes implicated in sepsis-associated organ injury, particularly lung injury. To address this gap, we obtained ChIP-chip data of sepsis-induced ARDS from the Gene Expression Omnibus (GEO) database. Furthermore, we corroborated our findings with an independent dataset and experiments.

Materials and Methods

Microarray Dataset Collection and Process

The GEO database (http://www.ncbi.nlm.nih.gov/geo/) was utilized to identify gene expression datasets related to ARDS, using the search terms "ferroptosis", "sepsis", "lung injury", and "microarray". The training set was represented by GSE32707, while GSE66890 served as the validation set. Detailed information on the GEO datasets is provided below: GSE32707 mRNA dataset: 13 sepsis + ARDS samples (ARDS) and 34 control samples (Control); GSE66890 mRNA dataset: 29 sepsis + ARDS samples (ARDS) and 28 sepsis samples (Control).

The data sets underwent quantile normalization using the "preprocessCore" package in R (version 4.2.0), and the probe IDs were converted to gene symbols based on platform information. Differential gene expression analysis was conducted on the two microarray datasets (GSE32707, GSE66890) using the "limma" package, with a filter criterion of logFC > 1 and p-value < 0.05.

Characteristics of Immune Cell Infiltration
The IOBR R software package was utilized to evaluate immune infiltration using our gene expression matrix. The FPKM values were employed to estimate 22 immune cell scores, and statistical significance was determined through 1,000 permutations with a p-value threshold of < 0.05. The resulting immune cell proportions were depicted in a bar chart.

**Identification of ferroptosis-Related DEGs**

Ferroptosis-associated genes (FAGs) were obtained from three distinct gene pools, namely the KEGG database, NCBI, and FerrDb database. The intersection of differentially expressed genes (DEGs) and FAGs was determined using a Venn diagram.

**Biological functions of ferroptosis-Related DEGs**

The DEGs associated with Ferroptosis were analyzed through enrichment and protein-protein interaction (PPI) analyses. The gene set functional enrichment analysis was conducted by utilizing the R package org.Hs.eg.db to perform Gene Ontology (GO) annotations of the genes. Additionally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway gene annotations were obtained through the KEGG rest API. Statistical analysis and graphical representations were generated using the R package clusterProfiler. The minimum and maximum gene set sizes were separately 15 and 500. Gene sets with p-value < 0.05 and FDR < 0.25 were deemed significant. The PPI and clustering analysis were operationalized with STRING (https://string-db.org/) and MCODE in Cytoscape 3.10.0. The diagnostic efficacy of hub genes was evaluated through the use of receiver operating characteristic (ROC) curves, employing the "pROC" package.

**Validation analyses**

The validation dataset (GSE66890) was utilized to authenticate the mRNA expression (via the "limma" package) and generate the ROC curve (using the "pROC" package) of the hub gene. The diagnostic precision was determined by comparing the area under the ROC (AUROC).

**Animals Experiment**

The Animal Ethics Committee of Qingdao Municipal Hospital (2021 – 118) granted ethical approval for the mice experiment, which adhered to the principles of animal protection and national experimental animal welfare ethics. Prior to the commencement of the experiment, the mice underwent a 12-hour fasting period.

Twelve adult male C57BL/6 mice, aged 6–8 weeks and weighing 20–25 g, were procured from Jinan Pengyue Experimental Animal Co., Ltd. The mice were randomly assigned to two groups, namely the Control group (n = 6) and the ARDS group (n = 6). To establish an ARDS mice model, LPS (5 mg/kg) was administered intraperitoneally (12). After 6 hours, the mice were euthanized, and their lung tissues were collected and stored at -80°C for subsequent analysis. In order to assess the histological alterations, the lung tissues were preserved in paraffin and subjected to haematoxylin and eosin (H&E) staining, followed
by observation under an optical microscope (Olympus Co., Ltd). The degree of lung injury was evaluated using a light microscope (400x) according to established protocols (13).

Quantitative reverse transcription-PCR analysis

Hub genes were verified by quantitative reverse transcription-PCR (RT-qPCR). In brief, total RNA was extracted using Trizol. RNA quantity was assessed using the Quantus fluorometer. (Thermo-Fisher). The amplified cDNA samples were reverse-transcribed using the Prime-Script RT Reagent Kit (TaKaRa, Tokyo, Japan). SYBR premix ExTaq (TaKaRa, Tokyo, Japan) was used for PCR reactions. The sequences of the primers for PCR were Lcn2: 5'-TGCAGGTGGTACGTTGTGG-3' (forward) and 5'-TGTTGTCGTCTTGAGGC-3' (reverse) and GAPDH (internal control): 5'-GGGAAACTGTGGCGTGAT-3' (forward) and 5'-GAGTGGGTGTCGCTGTTGA-3' (reverse). Gene expression was normalized with GAPDH and calculated using the 2^(-ΔΔCt) method.

Target gene prediction

The correlation between LCN2 gene and immune cell infiltration levels was analyzed by Pearson correlation analysis. The information about LCN2 gene was collected from GeneCard (http://www.genecards.org) databases. The LCN2 gene were predicted using human miRNA target gene prediction databases, StarBase 3.0 (algorithms Number ≥ 3, clipExpNum ≥ 2, and pancancerNum ≥ 5).

Statistical Analysis

Student’s t-test was used for continuous variables. Statistical analyses were performed using R (version 4.2.0) and SPSS 23.0 (SPSS, Inc., Chicago, IL, USA). p-value < 0.05 (two-sided) was considered statistically significant.

Results

Identification of DEGs in GSE32707 datasets

A total of 565 differentially expressed genes (DEGs) were identified from GSE332707, comprising 440 up-regulated and 125 down-regulated mRNAs, as illustrated by the volcano map in Fig. 1A. The top 6 DEGs, ranked by logFC, were identified, including the top-3 upregulated genes (DEFA3, DEFA1, and DEFA1B) and top-3 downregulated genes (TM4SF1, CPS1, and KRT8). Furthermore, Fig. 1B demonstrates significant differences in the infiltration of 6 immune cell markers (B cells naive, T cells CD4 naive, T cells CD4 memory resting, T cells follicular helper, monocytes, eosinophils). Among these, the T cells CD4 naive exhibited the most notable differential expression in the ARDS group. In addition, a Venn diagram analysis showed 30 overlapping DEGs (Fig. 1C), of which 20 overlapping DEGs were upregulated and 10 overlapping DEGs were downregulated.

Enrichment analysis of ferroptosis-related DEGs
In the GO-BP analysis (Fig. 2A), the top 10 enriched pathways were mainly cellular response to chemical stimulus, oxygen-containing compound, and cytokine stimulus, etc. In GO-MF analysis (Fig. 2B), the predominant pathways were oxidoreductase activity, CCR5 or CCR chemokine receptor binding. In GO-CC analysis (Fig. 2C), cytosol, extracellular exosome, and extracellular vesicle were the three major terms. The results of the enrichment analysis suggested that extracellular vesicles are involved in linking bacteria to ferroptosis. The outcomes of the enrichment analysis indicate that extracellular vesicles play a significant role in connecting bacteria to ferroptosis. The KEGG results (Fig. 2D) primarily demonstrate the activation of various pathways, including TNF signaling pathway, NOD-like receptor signaling pathway, and metabolic pathways. These results suggest that TNF signaling pathway and NOD-like receptor signaling pathway may have a crucial role in regulating ferroptosis in sepsis-induced ARDS.

**PPI network analysis of ferroptosis-related DEGs**

The PPI networks comprised of 30 nodes and 134 edges, as depicted in Fig. 3A. Notably, one of the 30 genes did not participate in the molecular network. The average node degree was calculated to be 8.93. By utilizing the "MODE" plug-in in Cytoscape, five hub genes (CTSB, LCN2, ZFP36, KLF2, IRF1), with the highest scores were identified, as illustrated in Fig. 3B. The AUROC values for CTSB and LCN2 in the diagnosis of sepsis-induced ARDS were 0.697 and 0.830, respectively. The AUROC values for ZFP36, KLF2, and IRF1 in sepsis-induced ARDS were 0.611, 0.516, and 0.706, respectively, as depicted in Fig. 3C. The findings indicate that LCN2 is a highly effective diagnostic marker for sepsis-induced ARDS.

**Validation of hub genes in the validation set and mice experiment.**

The validation set (GSE66890) comprised 57 cases, of which 28 were diagnosed with sepsis and 29 with sepsis + ARDS. Analysis of Fig. 4A revealed that LCN2 concentrations were significantly elevated in patients with ARDS compared to controls (p-value < 0.01), while CTSB, ZFP36, KLF2, and IRF1 did not exhibit significant differences. Furthermore, the hub gene, LCN2, demonstrated excellent diagnostic potential for sepsis-induced ARDS with an AUROC of 0.716 (Fig. 4B). Histological examination of lung tissue from sepsis mice was performed using HE staining. The examination of lung tissues in an ARDS model through HE staining revealed a disrupted alveolar structure, characterized by noticeable thickening of the alveolar walls and infiltration of inflammatory cells. Additionally, the lung injury score was significantly elevated following LPS stimulation compared to the Control group (Fig. 4C). Furthermore, the ARDS group exhibited increased RT-qPCR expression of Lcn2 (mice-origin) in lung sample

**Target LCN2 gene prediction**

The present study conducted a correlation analysis between LCN2 and immune cells using CIBERSORT. The results revealed a significant correlation between LCN2 gene expression and the differential expression of immune cell infiltration in GSE32707. Specifically, Eosinophils (r=-0.7396), T_cells_follicular_helper (r=-0.6949), B_cells_naive (r=-0.6283), T_cells_CD4_naive (r = 0.6060), T_cells_CD4_memory_resting (r=-0.5862), and Monocytes (0.5575) exhibited significant correlations with LCN2 gene expression (Fig. 5A). Furthermore, the data pertaining to the LCN2 gene, encompassing its compartmentalization, superpathways, and associated disorders, were procured from GeneCard. The
distribution of LCN2 was observed to be diffuse, predominantly extracellular in nature (as depicted in Fig. 5B). The findings indicated that LCN2 played a crucial role in the Innate Immune System, Cytokine Signaling in Immune system, and Transport of inorganic cations/anions and amino acids/oligopeptides pathways (as illustrated in Fig. 5C). Moreover, LCN2 was predominantly implicated in the etiology of neoplastic and inflammatory disorders, including but not limited to Breast Cancer, Pancreatic Cancer, Prostate Cancer, Inflammatory Bowel Disease, and Asthma (Fig. 5D). To delve deeper into the miRNA target gene of LCN2, established miRNA were scrutinized via StarBase 3.0, revealing a total of 5 target miRNAs, with miR-374b-3p exhibiting the highest target score.

Discussion

Empirically, approximately half of ARDS instances are attributed to sepsis (2). Several studies have indicated that ferroptosis has been shown to exacerbate both lung injury and cellular damage (5, 8). Correlations between FAGs and immune cells in sepsis-induced ARDS were determined, for the first time, in this study. Thirty DEGs related to ferroptosis were identified from both the GSE32707 and FerrDb datasets, with 20 exhibiting upregulated expression and 10 exhibiting downregulated expression in lung injury serum samples compared to normal serum samples. Subsequently, pathway enrichment analysis was conducted on these DEGs. The top enriched pathways were mainly cellular response to chemical stimulus, oxygen-containing compound, and cytokine stimulus, etc. The major pathways identified through GO-MF analysis were oxidoreductase activity, CCR5 or CCR chemokine receptor binding. GO-CC analysis revealed that cytosol, extracellular exosome, and extracellular vesicle were the three most significant terms. The KEGG results primarily indicated the activation of TNF signaling pathway, NOD-like receptor signaling pathway, and metabolic pathways, etc. Ultimately, a panel of five pivotal genes (CTSB, LCN2, ZFP36, KLF2, and IRF1) was identified through analysis of PPI networks. Notably, CTSB and LCN2 demonstrated the most significant clinical diagnostic potential. The validation of these findings through external validation and experimentation with mice further substantiated the reliability of LCN2 as a diagnostic marker. From a bioinformatics perspective, these results offer valuable insights for investigating the pathological mechanisms underlying sepsis-induced ARDS.

Ferroptosis, a form of programmed cell death, is characterized by its reliance on lipid ROS and iron. Ferroptosis is known to exert significant influence on the development of pulmonary conditions, including chronic obstructive pulmonary disease, acute lung injury, lung cancer, and asthma (14–17). Prior research has indicated a significant correlation between ferroptosis and immune response in various cell types, including vascular endothelial and epithelial cells, smooth muscle cells, and macrophages (18). This study has revealed the significant role played by T cells, monocytes, and eosinophils in the development of sepsis-induced ARDS. These cell types are recognized as key contributors to the inflammatory response within the respiratory system (19). However, further in-depth investigations are required to elucidate the mechanism of ferroptosis in relation to the amelioration of sepsis-induced ARDS in these cells.
Ferroptosis is a process that induces intracellular oxidation and lipid ROS accumulation by regulating glutathione peroxidase (20). Our study revealed that certain oxidative stress pathways, including those related to oxygen-containing compounds and oxidoreductase activity, were enriched with differentially DEGs. This finding strongly suggests that oxidative stress is a significant factor in the development of sepsis-induced ARDS. Furthermore, He et al. demonstrated that ferroptosis is involved in sepsis-induced ARDS in mice through the Nrf2 pathway (6). Furthermore, experimental evidence has demonstrated the involvement of ferroptosis and stimulated autophagy in sepsis-induced ARDS through the inhibition of mTOR signaling (21). The KEGG pathway analysis revealed a significant enrichment of CCR5 or CCR chemokine receptor binding. Previous studies have reported that the activation of the CCR5 signaling pathway in endotoxin-induced lung injury leads to increased neutrophil infiltration (22). Research has demonstrated that CCR5 regulates ferroptosis activity, thereby facilitating the progression of epithelial to mesenchymal transition in individuals undergoing kidney transplantation (23). Nonetheless, there exists a paucity of research studies that have established correlations between CCR5 signaling and ferroptosis in the context of ARDS, thereby presenting a promising avenue for future investigations. The outcomes of the KEGG analysis primarily indicated the activation of various pathways, including the TNF signaling pathway, NOD-like receptor signaling pathway, and metabolic pathways. However, the precise role of the TNF signaling pathway in ferroptosis remains a subject of controversy. It has been suggested that heightened TNF levels may trigger the activation of the NF-κB pathway, leading to the biosynthesis of cellular glutathione and consequent protection of fibroblasts from ferroptosis (24). The TNF/TNFR1/NF-κB signaling pathway has been shown to promote the expression of NO, potentially contributing to the sensitization of ferroptosis (25, 26). Additionally, the NOD-like receptor signaling pathway within the innate immune response has been found to impact the disease process of sepsis-induced ARDS (27, 28).

Previous research has indicated that a deficiency in the NLRP3 inflammasome in mice may reduce inflammation and ferroptosis by inhibiting the activation of the Keap1-Nrf2 pathway, ultimately alleviating cerebral ischemia/reperfusion injury (29). These findings suggest a potential interaction between the NOD-like receptor signaling pathway and ferroptosis in the context of sepsis.

The present study utilized gene microarray screening to identify key genes associated with ferroptosis-related DEGs, namely CTSB, LCN2, ZFP36, KLF2, and IRF1. Among these genes, LCN2 was found to hold the most potential for identifying and diagnosing sepsis-induced ARDS. LCN-2, a novel 198 amino acid secreted protein, belongs to a group of transporters responsible for circulating small molecules such as free fatty acids, LPS, and iron (30). Several studies have established the participation of LCN2 in diverse pathological states that impact multiple organs, including the liver, brain, renal system, lungs, and breast (31, 32). Furthermore, LCN2 has been linked to the initiation of both stress-induced behavioral responses and inflammatory pathways. The findings of An et al.’s experiments with mice demonstrated that the absence of LCN-2 led to the alleviation of acute lung inflammation triggered by LPS, as evidenced by the downregulation of genes associated with chemotaxis (33). Moreover, the upregulation of matrix metalloproteinase 9 in human neutrophil granulocytes can be facilitated by LCN2 (34), while LCN2 has been implicated in the exacerbation of acute lung inflammation and oxidative stress through the amplification of macrophage iron accumulation (35). However, the regulatory mechanism of the
upstream transcription factor and promoter analysis of LCN2 remains poorly understood. Our investigation has identified miR-374b-3p as the most prominent miRNA involved in the targeting of LCN2. The vast majority of our discoveries are predicated upon a comprehensive analysis of bioinformatics, which in turn requires further experimentation to verify the molecules and pathways governed by LCN2 in the context of sepsis-induced ARDS. Although our study did not satisfy the criteria for expression validation and ROC curve analyses, previous research has indicated significant involvement of the CTSB, ZFP36, KLF2, and IRF1 genes in pulmonary disease.

Our study has certain limitations that must be acknowledged. Although we used the peripheral blood of sepsis patients to verify the value of the above key genes, there was a lack of signal pathway-related mechanisms for further verification.

**Conclusion**

Our study has revealed the significant role of ferroptosis in the pathogenesis of sepsis-induced ARDS, which is closely associated with the modulation of inflammatory and oxidative responses through TNF and the NOD-like signaling pathway. Our results propose LCN2, a critical gene, as a potential therapeutic target for the management of sepsis-induced ARDS.

**Declarations**

**Author contributions**

Xiao Zhang drafted the manuscript. Yuan Ma participated in the bioinformatics analysis. Xiao Zhang and Weiwei Qin participated in the animal experiment. Yuting Dai, Fuguo Ma, and Lixin Sun provided the overall principle and direction of the study. All authors contributed to the article and approved the submitted version.

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**Competing interests**

The author(s) declare no competing interests.

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**Figures**

**Figure 1**

Identification of DEGs in GSE32707 datasets. (A) Volcano plot of DEGs in GSE32707 datasets. The red color meant upregulated genes, the blue color meant downregulated genes, and gray color meant no significant difference. (B) Differential expression of immune cells types between the ARDS and Control group. (C) Venn diagram of DEGs in the GSE32707 and ferroptosis genes with distinct colors representing different part. The left section (565 genes) represents DEGs unique to GSE32707, the right section (692 genes) is assigned to unique to ferroptosis genes, and the middle section (30 genes) refers to ferroptosis-related DEGs. * p-value < 0.05 versus Control. ** p-value < 0.01 versus Control.
Figure 2

Enrichment analysis of ferroptosis-related DEGs. (A) Top 10 GO enrichment pathway of ferroptosis-related DEGs at BP level. (B) Top 10 GO enrichment pathway of ferroptosis-related DEGs at MF level. (C) Top 10 GO enrichment pathway of ferroptosis-related DEGs at CC level. (D) Top 10 KEGG pathway analysis of ferroptosis-related DEGs.
Figure 3

PPI Network Analysis of Ferroptosis-Related DEGs (A) PPI network of differentially expressed Ferroptosis-Related DEGs. number of nodes: 30; number of edges: 134; average node degree: 8.93; avg. local clustering coefficient: 0.641; expected number of edges: 62; PPI enrichment p-value: 8.88e-16. (B) Reconstructed subnetwork of top 10 hub genes based on the PPI network. Yellow color represents the top 5 hub genes. (C) The ROC curve of the top 5 hub genes to diagnosis ARDS.
Figure 4

Validation of hub genes in the validation set and mice experiment. (A) Differential expression of the top 5 hub genes between ARDS and Control group in GSE66890. (B) The ROC curve of the top 5 hub genes in the Validation Set. (C) Histological changes of lung tissues after LPS treatment detected by HE staining and viewed at 400x magnification. Scale bar, 50 μm; The lung injury score was calculated according to HE staining; Relative mRNA expression of Lcn2 after LPS treatment. Results are the mean ± SD. n=6. ** p-value< 0.01 versus Control.
Figure 5

Target LCN2 gene prediction. (A) The correlation between LCN2 gene and immune cell infiltration in GSE32707 (B) A pie chart of compartment distribution of LCN2 according to GeneCard. (C) A pie chart of superpathway of LCN2 according to GeneCard. (D) A pie chart of disorders of LCN2 according to GeneCard. (E) The targeted miRNA for LCN2 according to the information from StarBase 3.0.